

Isolation of *Coccidioides immitis* F Antigen by Immunoaffinity Chromatography with Monospecific Antiserum

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Detection of antibody to *Coccidioides immitis* F antigen is of proved value in the diagnosis of coccidioidomycosis. This antibody is demonstrable by use of an immunodiffusion assay with reference coccidioidin antigen and antiserum to *C. immitis*. Using a combination of lectin affinity and immunoaffinity chromatography, we isolated the F antigen from coccidioidin and prepared monospecific antibody to the purified antigen. The availability of these reagents will enable the development of a sensitive and specific assay for detecting serologic reactivity to this antigen.

Coccidioidomycosis is a mycotic disease acquired by inhalation of mycelial-phase arthroconidia of *Coccidioides immitis*. Clinical disease ranges from a benign, self-limited infection to a severe, progressive, and often fatal mycosis involving pulmonary and extrapulmonary tissues (5).

One of the most useful serologic tools for confirming the clinical diagnosis of coccidioidomycosis is the demonstration of complement-fixing (CF) antibody, a humoral response which is detected in the majority of patients within 3 months of clinical onset (17, 18). Although the CF assay has an acceptable level of sensitivity, cross-reactions are known to occur with sera of patients with other mycoses, particularly those with histoplasmosis or blastomycosis (2, 14).

In 1963, Huppert and Bailey (8) introduced an immunodiffusion (ID) assay wherein coccidioidin (CDN), prepared as a broth culture filtrate of mycelia, was titrated against sera of patients to yield a single precipitin band which correlated with the results obtained in the CF test. This procedure was designated the IDCF assay, and the precipitinogen is referred to as the F antigen (10). It has not yet been proved that the F antigen is identical to the CDN component which reacts with antibody in the CF assay; however, the strong correlation between the results of the two assays (7, 10, 11, 20) and the finding that both the F and CF antigens are labile to heating at 56°C for 30 min (10, 17) support this presumption.

The results of the IDCF assay are 100% specific (8-10, 14). That is, the complete fusion of the precipitin band formed between the antibody of the patient and CDN with the F precipitin band formed between the reference CDN/anti-CDN system establishes the presence of antibody to *C. immitis*. This high level of specificity of the IDCF assay and the documented clinical usefulness of detecting this antibody response prompted studies in our laboratory to purify the F antigen from CDN. The results are detailed in the present report.

MATERIALS AND METHODS

Antigen. The CDN preparation used to isolate the F antigen was obtained as the soluble broth culture filtrate of

mycelia grown for 3 days in glucose-yeast extract medium at 33°C (4). The presence of the F antigen in this CDN preparation was established by the IDCF assay, in which reference reagents purchased from Nolan Laboratories (Atlanta, Ga.) were used.

Lectin affinity chromatography. Affinity chromatography was performed on columns (2.5 by 10 cm) containing concanavalin A (ConA) covalently linked to Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.). CDN (8 mg) was dissolved in 0.01 M Tris-buffered saline (pH 7.2) containing 10⁻⁴ M CaCl₂ and 10⁻⁴ M MgCl₂ and applied to columns preequilibrated with buffer. Nonbound components were eluted at a descending flow rate of 15 ml/h. After the A₂₈₀ of the effluent returned to base line, bound components were eluted in buffer containing 0.2 M methyl α-D-mannopyranoside, a competitive inhibitor of ConA binding (6). The effluent and eluate fractions were dialyzed against distilled water and then lyophilized.

Immunoaffinity chromatography. Solid-phase immunoadsorption testing was performed on columns containing ammonium sulfate-precipitated goat immunoglobulin (3) covalently coupled to Sepharose 4B. Coupling was performed by incubation of 75 mg of immunoglobulin dissolved in 0.1 M bicarbonate buffer (pH 8.3) with 5 g of cyanogen bromide-activated Sepharose 4B (Sigma). The suspensions were tumbled overnight at 4°C and then washed by centrifugation in bicarbonate buffer. Nonreactive groups were blocked with 0.2 M glycine, and the coupled gels were washed three times with alternating cycles of 0.1 M acetate buffer (pH 4.0) and 0.1 M borate buffer (pH 8.0), each containing 1 M NaCl. The immunosorbent gels were transferred to columns (2.5 by 10 cm) and equilibrated with 0.0067 M phosphate-buffered saline (PBS, pH 7.2) containing 0.02% sodium azide as a preservative. Antigen (2 mg [dry weight] in 0.5 ml of PBS) was dissolved in PBS and applied to the columns. Nonbound components were eluted at a descending flow rate of 12 ml/h. After the A₂₈₀ of the effluent returned to base line, the column bound fraction was desorbed with 0.1 M glycine hydrochloride (pH 2.3) or, where indicated, with 8 M urea in 0.15 M sodium bicarbonate (pH 7.0). The eluate and effluent fractions were dialyzed against distilled water and then lyophilized.

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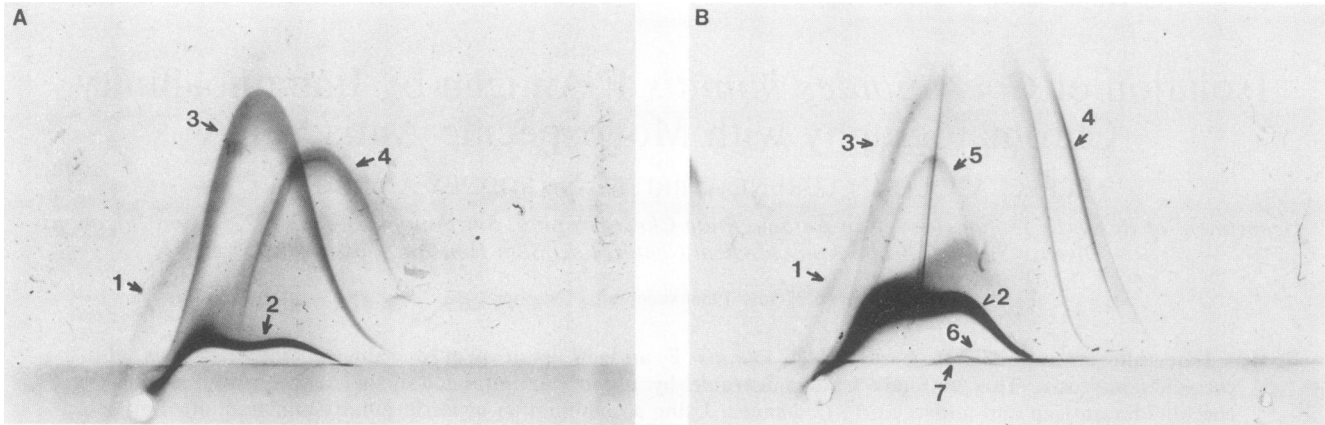


FIG. 1. Antigenic composition of CDN as assessed by 2D-IEP against goat anti-CDN. (A) Unheated CDN (100 µg) was assayed against an immunoglobulin concentration of 325 µg/cm² of gel. (B) Unheated CDN (600 µg) against 810 µg of immunoglobulin per cm² of gel.

Immunization. Antigen (or the precipitin arc obtained in two-dimensional immunoelectrophoresis [2D-IEP]) was suspended in PBS and mixed with an equal volume of complete Freund adjuvant and then injected intramuscularly into goats. The goats were boosted at monthly intervals with antigen (or precipitin arc) in incomplete adjuvant. The immunoglobulin fraction of sera was isolated as described previously (3).

2D-IEP. The techniques of 2D-IEP were performed according to methods detailed in earlier reports (3, 4). Hyperimmune goat anti-CDN was the same antiserum used previously (3, 4) and, before use in this study, was shown to be reactive with the F antigen, as assessed in the IDCF assay.

Immunoblot analysis. Antigens were diluted in buffer containing 1% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, 0.001% bromophenol blue, 0.0625 M Tris hydrochloride, and 5% sucrose and then heated for 20 min at 37°C. Reference IDCF antigen (3 mg) was electrophoresed through a 3 to 27% gradient sodium dodecyl sulfate-polyacrylamide gel (Integrated Separation Systems, Newton, Mass.) in a discontinuous buffer system as described by Laemmli (15). Electrophoresis was performed at 40 mA per gel until the ion front reached the gel bottom, as judged by the migration of bromophenol blue.

Immunoblotting of the gels was performed by a modification of the method of Towbin et al. (19). Briefly, antigens were electrophoretically transferred to a nitrocellulose membrane (NCM; 0.20 µm pore size) at 1 A for 2 h in a Transphor Unit (Hoefer Scientific Instruments, San Francisco, Calif.). The NCM was blocked for 2 h at 37°C with Bovine Lacto Transfer Technique Optimizer (BLOTTO) consisting of 5% (wt/vol) nonfat dry milk diluted in PBS containing 0.01% Antifoam A (Sigma) as described by Johnson et al. (12). After blocking, the NCM was incubated overnight with primary antibody diluted 1:25 in BLOTTO. The NCM was washed three times in BLOTTO, and the individual lanes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy and light chains; Cooper Biomedical, Inc., West Chester, Pa.) or alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G (heavy and light chains; Kirkegaard and Perry Laboratories, Gaithersburg, Md.), depending upon the primary antibody used. After a 1-h incubation at 25°C, the blots were washed three times in PBS and developed with 5-bromo-4-chloro-3-indolyl-phosphate-nitroblue tetrazolium (BCIP/NBT Phosphatase Substrate, Kirkegaard and Perry) diluted 1:10 in 0.1

M Tris buffer. The nonspecificity of reactants was ruled out by selective exclusion of antigen, primary antibody, or conjugate.

RESULTS

Antigenic analyses of CDN by 2D-IEP. The antigen composition of the CDN preparation used in the isolation of the F antigen was evaluated by 2D-IEP against reference goat anti-CDN (Fig. 1). Under conditions in which the concentrations of CDN and goat anti-CDN were varied, a total of seven precipitinogens, designated antigens Ag1 through Ag7, were demonstrable (Fig. 1A and B). Of these, three (Ag3, 5, and 6) were labile to heating at 56°C for 30 min (data not shown). Because the F antigen is heat labile (10) and can be detected as a precipitinogen, attention was focused on determining which of these three antigens was reactive in the IDCF assay.

Fractionation of CDN by ConA chromatography. Affinity chromatography of CDN on ConA-Sepharose produced an effluent fraction containing only Ag3, 4 and 7, as assessed by 2D-IEP against goat anti-CDN (Fig. 2). The peak height of Ag3 was significantly reduced, compared with its height in unfractionated CDN, a result that is consistent with the

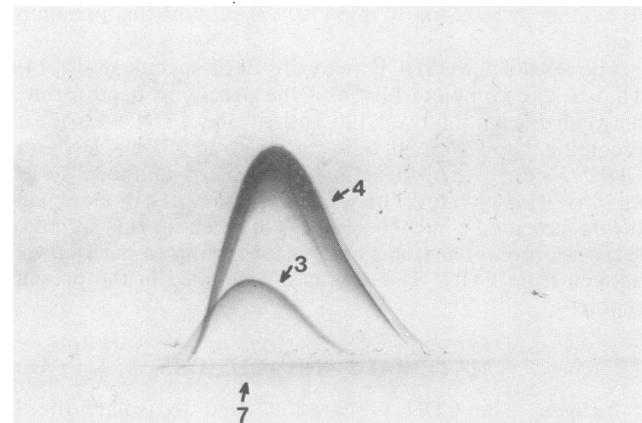


FIG. 2. Antigenic analyses of the ConA effluent fraction (100 µg) as assessed by 2D-IEP against goat anti-CDN (810 µg of immunoglobulin per cm² of gel).

detection of this antigen in large amounts in the ConA eluate fraction (data not shown).

Reactivity in the IDCF assay was demonstrable with both the ConA eluate and effluent fractions. This result, together with the finding that Ag3 was the only heat-labile precipitinogen detected in the two fractions, suggested that Ag3 was the F antigen.

Fractionation of the ConA effluent by immunoaffinity chromatography. Attempts to purify Ag3 from the ConA effluent or eluate fractions on molecular-sieve or ion-exchange columns were not successful; i.e., Ag3 coeluted with one or more other CDN components. Nor were we able to purify (or enrich for) Ag3 by treatment of the fractions with trypsin or protease (10 $\mu\text{g}/\text{mg}$ of antigen, 1 h, 37°C) or sodium metaperiodate (0.05 M, 18 h, 4°C). Rather, Ag3 was susceptible to treatment with proteolytic enzymes and to periodate oxidation, as evidenced by a loss of precipitating reactivity in 2D-IEP.

On the basis that the ConA effluent fraction contained Ag3 and only two other precipitinogens (Ag4 and 7) and that the former was labile to heating whereas the latter two antigens were heat stable, an indirect approach was used to isolate Ag3. Namely, goat antiserum was prepared against the heat-treated ConA effluent and then used to adsorb the heat-stable antigens by immunoaffinity chromatography. Initial studies indicated that heat treatment of the ConA effluent at 100°C for 30 min completely destroyed the antigenicity of

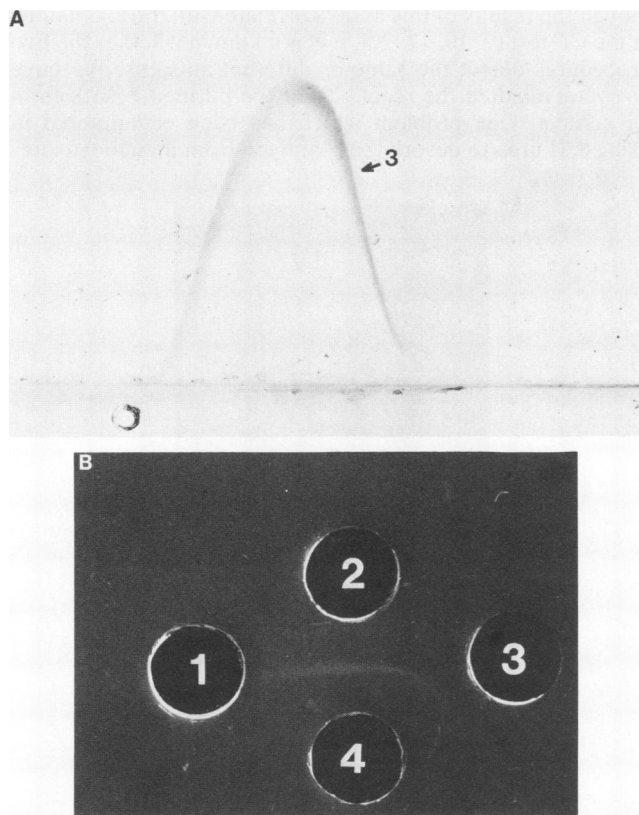


FIG. 3. Antigenic analyses of the fractions obtained by immunoaffinity chromatography of the unheated ConA effluent with goat antiserum to the heat-treated ConA effluent. (A) 2D-IEP of the column effluent (300 μg) against goat anti-CDN (810 $\mu\text{g}/\text{cm}^2$ of gel). (B) Reactivity of the column eluate (well 1) and effluent (well 3) in the IDCF assay. Well 2 contains reference IDCF antigen; well 4 contains reference IDCF antiserum.

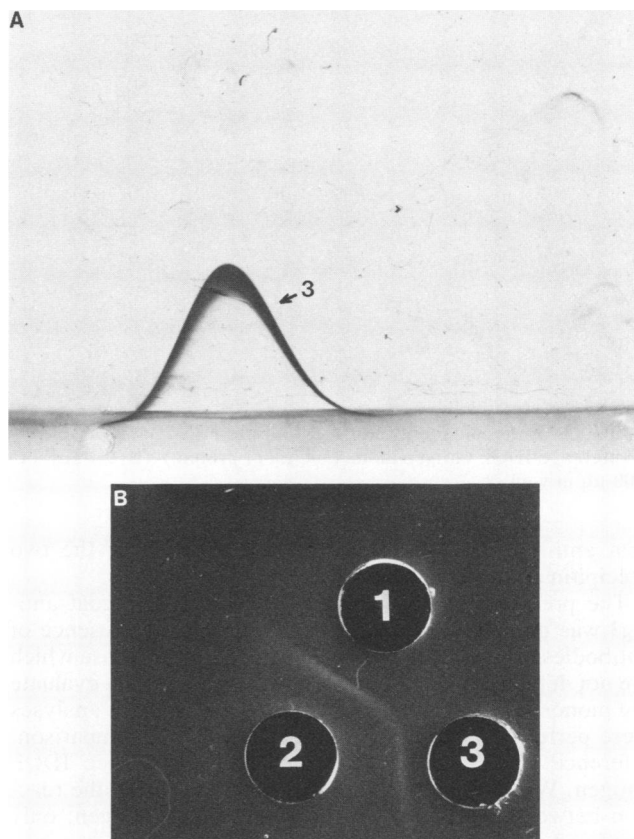


FIG. 4. Antibody specificity of goat anti-Ag3. (A) 2D-IEP of unfractionated CDN (300 μg) against goat anti-Ag3 (400 $\mu\text{g}/\text{cm}^2$ of gel). (B) Reactivity of goat anti-Ag3 (well 3) in the IDCF assay. Well 1 contains reference IDCF antiserum; well 2 contains reference IDCF antigen.

Ag3 as assessed by lack of a precipitin peak in 2D-IEP. This procedure also destroyed the immunogenicity of Ag3; i.e., goat antiserum prepared against the heat-treated ConA effluent fraction was without demonstrable reactivity with Ag3 when evaluated by line IEP (not shown).

Antiserum to the heat-treated ConA effluent proved effective in adsorbing Ag4 and 7 from the unheated ConA effluent as evidenced by the detection of only Ag3 in the effluent fraction from the immunoabsorbent column (Fig. 3A). When analyzed by the IDCF assay, the column effluent and not the eluate fraction contained the F antigen (Fig. 3B). Together, these results establish the identity of Ag3 with the F antigen.

Production of monospecific antiserum. Studies were next directed toward preparing monospecific antiserum to Ag3 by use of the column effluent fraction obtained by solid-phase immunoabsorption. Since this fraction may have contained other components which were not detectable by 2D-IEP, we used the precipitin arc obtained in 2D-IEP of Ag3 against goat anti-CDN as the immunogen.

Immunization of a goat over a 5-month period with the immune-complexed antigen induced antiserum that was monospecific for Ag3 as assessed by 2D-IEP (Fig. 4A). The same antiserum was reactive with the F antigen in the IDCF assay (Fig. 4B) and, when used in 2D-IEP against the reference IDCF antigen, detected a single precipitinogen (Fig. 5A). The antigenic identity of this reference IDCF precipitinogen with Ag3 was established by tandem IEP. 2D-IEP of the reference antigen in tandem with CDN against

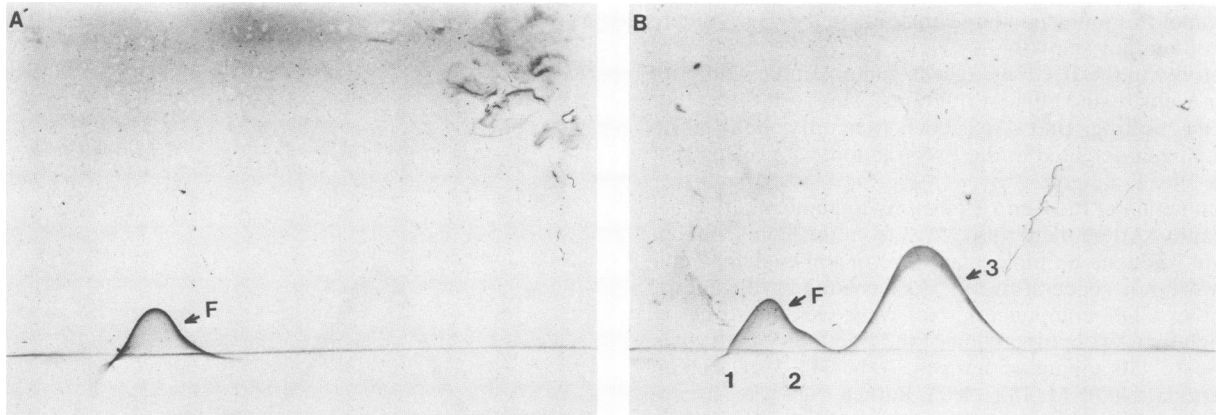


FIG. 5. Antigenic identity of Ag3 and the F antigen as assessed by tandem 2D-IEP against goat anti-Ag3 (320 $\mu\text{g}/\text{cm}^2$ of gel). (A) 2D-IEP of reference IDCf antigen alone (300 μg of protein). (B) 2D-IEP of reference IDCf antigen (300 μg of protein in well 1) in tandem with CDN (100 μg in well 2).

goat anti-Ag3 resulted in the complete fusion of the two precipitin peaks (Fig. 5B).

The preceding results provided evidence that goat anti-Ag3 was monospecific but did not rule out the presence of antibodies to other *C. immitis* antigens, notably those which are not detectable as precipitinogens. To more fully evaluate the monospecificity of this antiserum, immunoblot analyses were performed with goat anti-Ag3 and, for a comparison, reference IDCf antiserum, each against reference IDCf antigen. Whereas multiple bands were detected in the reaction between reference IDCf antiserum and antigen, only one band was detected with goat anti-Ag3 (Fig. 6).

Isolation of the F antigen by use of goat anti-Ag3. The monospecificity of goat anti-Ag3 and its reactivity in the IDCf assay prompted studies to evaluate the utility of this antiserum for use as a ligand for isolating the F precipitinogen. Immunoaffinity chromatography of the reference IDCf antigen (3 mg) on columns containing goat anti-Ag3 resulted in the complete adsorption of the F antigen; i.e., the effluent fraction was without reactivity in the IDCf assay (Fig. 7A). Desorption of the bound component(s) with 8 M urea yielded the F precipitinogen, in biologically active form, as established by the reactivity of the eluate fraction in the IDCf assay (Fig. 7A). To further verify that antigenic determinants were not altered during the desorption procedure, the eluate fraction was electrophoresed in tandem with CDN against goat anti-Ag3 (Fig. 7B). Although the peak height of the desorbed F antigen was reduced, compared with that in the unfractionated IDCf antigen preparation (Fig. 5A), the fusion of the two precipitin peaks confirms that antigenic determinants remained intact. These results establish the efficacy of goat anti-Ag3 as a biospecific ligand for the F antigen.

DISCUSSION

The clinical value of detecting antibody to the F precipitinogen is well established (9–11, 13, 14, 20). Using a combination of affinity and immunoaffinity chromatographies, we isolated the F antigen from CDN and prepared monospecific goat antiserum to the purified antigen. The availability of this antiserum and the purified F antigen will enable the development of a sensitive and specific immunoassay for detection of this serodiagnostic antibody response.

The purification of the F antigen was accomplished by a circuitous approach, i.e., production of an antiserum to

heat-stable components in the ConA effluent fraction and the removal of these components by immunoaffinity chromatography. Nonetheless, sufficient quantities of the purified F antigen were obtained by this procedure for use as an immunogen and the resulting antiserum was proven to be an effective ligand for isolating the F antigen directly from CDN.

The IDCf assay is a highly specific procedure for establishing the diagnosis of coccidioidomycosis (8–10, 14). Although the results of this assay correlate with those obtained in the CF test (7, 10, 11, 20), it is not known whether the two procedures detect the same or different antigens. We have not yet evaluated the reactivity of the purified F antigen in CF assays. One problem which has been encountered in using 8 M urea to desorb the F antigen from immunoabsorb-

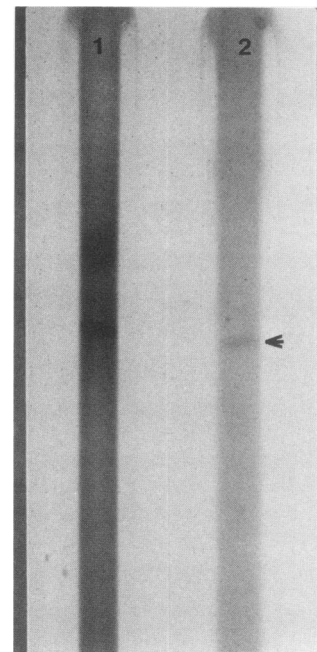


FIG. 6. Monospecificity of goat anti-Ag3 as assessed by immunoblotting. Reference IDCf antigen (3 mg as protein) was electrophoretically transferred to nitrocellulose membrane and then reacted with reference IDCf rabbit antiserum (lane 1) or goat anti-Ag3 (lane 2).

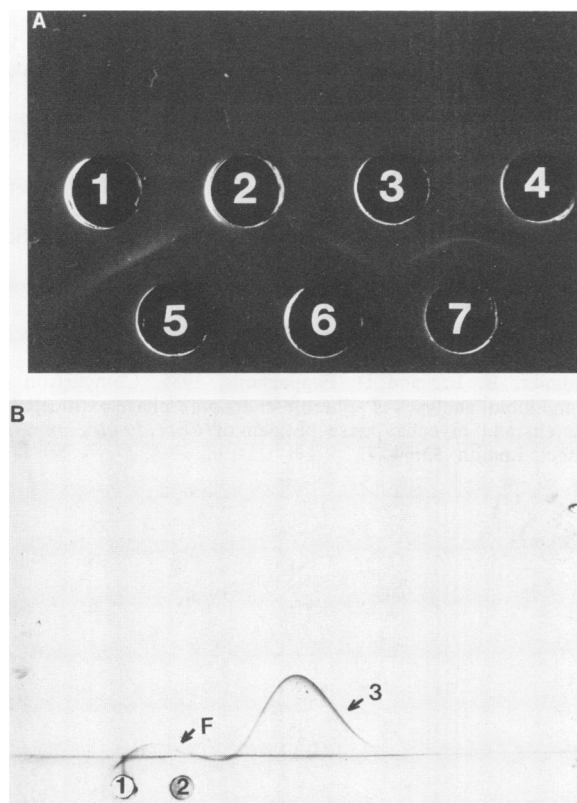


FIG. 7. Isolation of the F antigen from reference IDCF antigen by immunoaffinity chromatography with goat anti-Ag3. Reference IDCF antigen (0.5 ml; 3 mg as protein) was applied to immunoabsorbent columns containing goat anti-Ag3. The nonbound components were eluted in PBS; the bound fraction was desorbed with 8 M urea. After dialysis and lyophilization, the column eluate and effluent fractions were resuspended in 0.25 ml of PBS. (A) Reactivity of the eluate in the IDCF assay. Wells 1 and 3 contain unfractionated IDCF antigen; well 2 contains the column effluent fraction; well 4 contains the eluate fraction; wells 5, 6, and 7 contain reference IDCF antiserum. (B) 2D-IEP of the eluate (well 1) in tandem with CDN (100 μ g in well 2) against goat anti-Ag3 (320 μ g/cm² of gel).

ent columns is the desorption of some of the goat anti-Ag3. These antibodies are likely to be complexed with antigen and would interfere, therefore, with the CF assay by binding complement. To circumvent this problem, we are currently purifying the immunoglobulin G fraction of goat anti-Ag3 by immunoabsorption with protein A (from *Staphylococcus aureus*), a procedure which can similarly be used to remove goat antibody from the desorbed fraction. The presence of goat antibody in the eluate also complicates analyses of the F antigen in procedures such as enzyme-linked immunosorbent assays. Preliminary assays have been performed, however, to evaluate the sensitivity and specificity of the desorbed F antigen by double immunodiffusion in agarose against sera of patients with systemic mycoses. Of 10 patients with active coccidioidomycosis, all showed precipitin reactivity, whereas none of 8 patients with histoplasmosis or 3 patients with blastomycosis reacted. Although these analyses have been limited to a small number of patients, they indicate that the specificity, as well as the sensitivity, of the F antigen is retained after isolation by immunoaffinity chromatography.

In a recent study, Zimmer and Pappagianis (21) analyzed

the reactivity of antigens in CDN and spherulin with sera of coccidioidomycosis patients by the technique of Western immunoblotting. These investigators reported that a heat-labile 48-kilodalton (kDa) antigen was reactive with immunoglobulin G antibody in sera of patients which were positive by the IDCF assay. Using a similar approach, Calhoun et al. (1) demonstrated reactivity of a 45- to 48-kDa antigen with IDCF-positive sera of coccidioidomycosis patients. We do not yet know if the F antigen isolated in this study is similar or identical to the 48-kDa antigen reported by Zimmer and Pappagianis (21) or the 45- to 48-kDa antigen reported by Calhoun et al. (1). Although we have used the technique of immunoblotting to assess the monospecificity of antiserum prepared against the F antigen, we do not believe that this technique is well suited for analyses of the F antigen. This conclusion is based upon our finding that the F antigen is labile to the conditions used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis which, as pointed out by Zimmer and Pappagianis (21), are required for optimal resolution of CDN components in immunoblots. Although 2D-IEP is less sensitive than immunoblot analyses and is limited to detection of precipitinogens, this technique offers the advantage of detecting antigens in their native conformation (16).

ACKNOWLEDGMENT

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